APPENDIX A

RESPONSE BY THE INVENTOR, Dr. JAMES KAPUT, TO REQUEST FOR INFORMATION BY THE EXAMINER

Response to paragraphs 9, 10, 11 and 13 (examiner's request to provide "explanation of publication's contribution to description of prior art.")

NutraGenomics combines molecular genetic technologies and approaches to analyze the response to well-balanced diets (nutrition) in inbred mice with different susceptibilities to disease (comparative genomics).

A. **Genetics – Experimental use of Comparative Genomics**

Comparative genetics has been an active field for almost 100 years. Much of the work was initiated by C.C. Little and continued by scientists at the Jackson Laboratory. Finding the "first" article describing such approaches is problematic because the field of mouse genetics began in the early 1900s.

The effect of an individual gene on physiology differs depending upon the genetic background: A review of Coleman's classic work on two single gene mutations, obese (*ob*) and diabetes (*db*). His work showed that these genes affect different inbred strains differently. Specifically, BL/6 inbred mice respond to *ob* and *db* genes by expanding beta cell populations and the increased cell numbers eventually produce enough insulin to control hyperglycemia. In BL/Ks inbred mice carrying either *ob* or *db*, beta cell expansion doesn't occur and full blown diabetes develops. Geneticists use the term epistasis for this phenomenon: epistasis is the interaction between nonallelic genes, especially when one suppresses the other. The genetic background (i.e., in this case, BL/Ks) alters expression or effect of individual mutant genes. Alternatively, BL/Ks alleles are epistatic to *ob* or *db*.

1. **Hoag, WG. 1963. Spontaneous Cancer in Mice. Annals NY Acad. Sci 108, 805 – 831.**

Strain differences reveal information about diseases processes: The Hoag paper is a comprehensive review of all literature related to cancer in inbred strains dating to the early part of the 1900s, and demonstrates that strain specific differences exist. That is, different genotypes (collections of alleles of genes) are unique to a given strain, which are produced by continual brother X sister in each generation. Different combinations of alleles differ among different strains (genotypes) giving rise to different genetic susceptibility to disease. Hoag cites studies that compared strains in one study.

2. **Coleman, DL. 1978. Obese and Diabetes: Two Mutant Genes Causing Diabetes-Obesity Syndromes in Mice. *Diabetologia* 14, 141 – 148 (1978) A review article.**

The Hoag and Coleman reviews demonstrate the importance of not relying on one strain (genotype) to identify disease genes – any given strain (genotype) might "block" the expression of the disease gene. Comparison between different genotypes should therefore permit the identification of genes (more precisely alleles) that contribute to or cause differences between strains.

B. Importance of Diet Consistency on Model Studies and Disease - Experimental

The experimental design of our studies controls for the composition (i.e., amount of dietary fat, carbohydrate, protein, vitamins, minerals, fiber, etc) and the nutritional state of the animal (fed vs fasted). These papers demonstrate the importance of using well defined diets for the experiments.

Altering diet changes incidence of diabetes in NOD mice. Much of the research conducted in laboratory animal strains is done with chow diets – diets from a commercial source made from least expensive, unrefined material. Colman et al showed that a semi-defined diet – one with known quantities and qualities of dietary constituents – produced different incidence of diabetes in NOD mice. The results are dramatic: animals fed chow diets had a much greater incidence of diabetes compared to mice fed the semi-defined diets.

1. **Coleman, DL. Kuzava, JE., and Leiter, EH. 1990. Effect of Diet on Incidence of Diabetes in Nonobese Diabetic Mice.** *Diabetes* **39, 432 – 436.**

Constituents in Diets Vary, unless Rigorously Controlled. The two papers cited below are examples of research on the variability of chow diets. A closed-formula diet reports the dietary ingredient, but the concentrations are not stated and may vary from batch to batch. Open-formula diets should report ingredients, concentrations, and these should not vary from batch to batch.

Both of these studies demonstrate a significant variation in constituents and concentrations between diets and/or lots. Note, that this variation may not matter for certain biological experiments, but will be very important for studies of gene regulation – particularly when comparing across different feeding studies done at different times. We use diets constructed by Research Diets Inc (New Brunswick, NJ) and they are rigorously controlled. In addition, we also have adopted a 4% corn oil diet as a control in all feeding experiments, allowing for meaningful cross experiment comparisons.

2. **Lardinois, CK, Caudill, T., Starich, GH. 1989. Dissimilar Fatty Acid Composition of Standard Rat Chow.** *Am. J.Med.Sci.* **298, 2305 - 308.**

3. **Thigpen, JE, Setchell, KDR, Ahlmark, KB, Locklear, J., Spahr, T, Caviness, GF, Goelz, MF, Haseman, JK, Newbold, RR, and Forsythe, DB 1999. Phytoestrogen Content of Purified, Open- and Closed-Formula Laboratory Animal Diets.** *Laboratory Animal Sci.* **49, 530 – 536.**

These three papers demonstrated (i) that diet alters the expression of genetic information to cause disease and (2) that chow based diets are highly variable.

C. Nutrient Control of Gene Expression

Our underlying assumption is that subsets of genes regulated by diet are involved in disease processes.

Gene Expression – Single Genes: Many researchers investigate the nutritional regulation of specific genes. The vast majority of these studies pre-select the gene of interest based upon historical or biochemical rationale. For example, Goodridge's lab published 23 papers on various aspects of fatty acid and TCA cycle enzymes and then continued that work through the cloning era. The article cited above appears to be the first paper using molecular biology methods to examine expression of a single gene in response to dietary changes.

For the purposes of the patent, changes in expression of a single gene rarely produce *chronic* diseases, such as obesity, diabetes, or cardiovascular disease. Over-expression of certain oncogenes alters phenotype (of course), but these are typically caused by mutations, not naturally occurring allelic variants.

1. **Morris SM Jr, Nilson JH, Jenik RA, Winberry LK, McDevitt MA, Goodridge AG. 1982. Molecular cloning of gene sequences for avian fatty acid synthase and evidence for nutritional regulation of fatty acid synthase mRNA concentration.** *J Biol Chem* **257, 3225 - 3229**

The transition to high throughput methods to isolate previously unidentified diet regulated genes: With the advent of differential screening technologies, it was possible to identify and characterize previously unknown genes regulated by dietary variables. The first two papers using this approach were for isolating genes regulated by dietary lipids (our 1993 paper) and by dietary zinc – both were published in January 1993. The purpose of Cousins' paper was to isolate Zn transporter genes. Cousins group went on to use differential display technologies to identify other Zn regulated genes (as did we, but we did not publish the work). Modern techniques use microarray analyses to estimate the abundance of messenger RNA and the differences in abundance between dietary treatments or disease and normal phenotypes.

2. **Shay NF, Cousins RJ. 1993. Cloning of rat intestinal mRNAs affected by zinc deficiency** *J. Nutrition* **123, 35 - 41**

Dietary chemicals control gene expression. Many reports in the late 1980s and early 1990s showed regulation of gene expression by diet, but many interpreted the regulation as going through hormonal pathways and signaling (e.g., insulin and glucagons
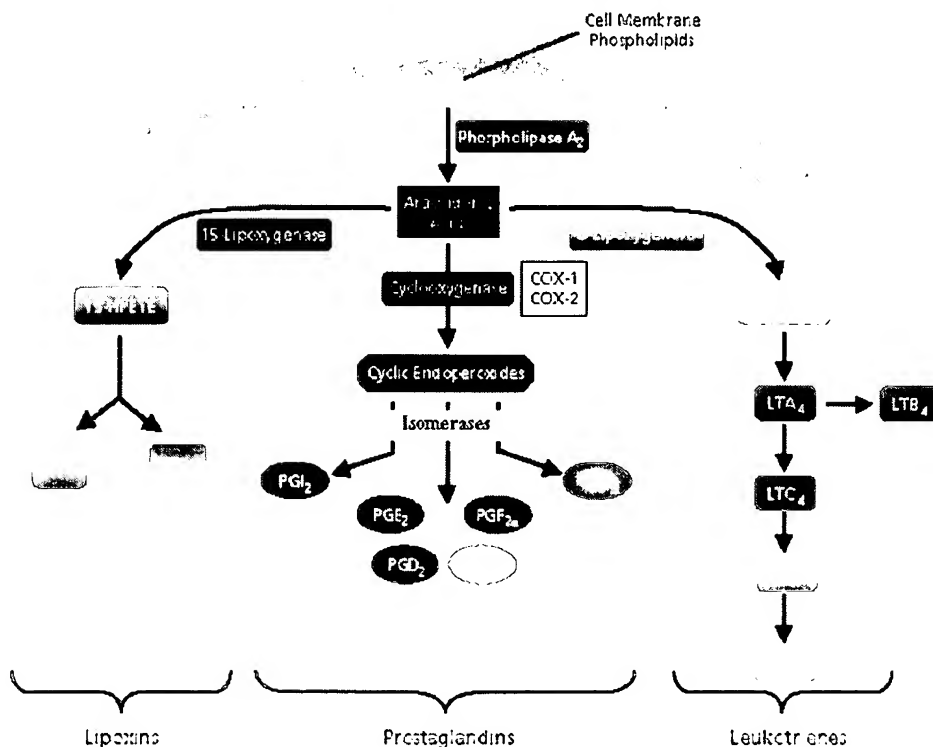

Figure 1: Arachidonic Acid metabolism (from Sigma Aldrich)

regulation). The peroxisome proliferator activated receptor (PPAR) story broadened the possibilities of gene regulation to include specific dietary chemicals. PPARs were discovered by Gustafsson's group in Stockholm and were dubbed orphan receptors - that is, their activation ligands were unknown when they were first cloned. These receptors belong to a nuclear receptor superfamily, a group of structurally related, ligand-dependent transcription factors. There are about 50 such similar genes and the ligands for many are still uncharacterized. Evans' group identified the first ligand for the nuclear receptor, PPAR – gamma (PPAR-γ), an arachidonate metabolite, 15-deoxy-delta 12, 14-prostaglandin J2. Lehmann published essentially the same results in the same issue of *Cell*. Figure 1 shows the arachidonic acid pathway, although it doesn't include the J2 derivative. This chemical activates PPAR-γ and induces adipogenesis. Many of the orphan receptors are likely to be activated by ligands derived from the diet. For example, LXR is a liver specific oxysterol receptor that regulates cholesterol metabolism, and FXR (farnesoid X receptor) is a regulated by bile acids

3. **Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma.** *Cell* **83, 803 - 812**

4. **Kliewar, SA, Lenhard, JM, Willson, TM, Patel, I, Morris, DC, and Lehmann, JM. 1995. A Prostaglandin J_2 Metabolit Binds Peroxisome – Activated Receptor γ and Promotes Adipocyte Differentiation.** *Cell* **83, 813 – 819**

These 4 papers indicated that diets and specific chemicals in food could alter gene expression – the basis of our experimental approach. We added a specific step to our protocols – removal of food for 12 hours before allowing the mice to eat for 2 hrs – to synchronize the nutritional state. Mice are killed in the fed or fasted state. The feeding protocol is similar to that adopted for human blood tests: almost all reports of physiological measurements (cholesterol levels, LDL levels etc) are from blood drawn after an overnight fast. This allows comparisons among different individuals or in the same individual following dietary or medical treatments.

D. Diet Effect on Disease - Experimental

Numerous publications look at the effect of diet on health in mice or laboratory animals. The first study showing that dietary fat promoted tumors in laboratory animals was published in the 1940s. Since then, many studies have demonstrated an effect of diet on disease in laboratory animals for virtually every chronic diseases.

Diet influences health: Based upon many historical reviews, Tannenbaum authored the first paper describing the effects of high fat diets on cancer. This one paper spawned hundreds (or more) of similar studies, but in general, the end point was cancer (or some other chronic disease). That is, the mechanisms whereby diet or dietary chemicals altered phenotype from normal to disease were not analyzed. A more recent review of the effect of diet on health was published in 2002.

1. **Tannenbaum, A. 1942. The Genetics and Growth of Tumors: III. Effects of a High Fat Diet.** *Cancer Research* **2, 468 – 475. Genetic Basis of Disease**

2. **Willett WC. 2002. Balancing life-style and genomics research for disease prevention.** *Science* **296, 695 – 698 A review.**

E. Genetic Effects on Disease

The foundation for the modern approach to the analyses of genetic basis of disease was proposed by Botstein, White, Skolnick, and Davis in 1980. Although methods for

analyses of polymorphisms have improved, they are all based upon the same concept: associate variations in sequence in regions of chromosomes to complex single gene defects (cystic fibrosis) or complex phenotypes. In most of the early analyses, the identity of the disease gene was not known. Rather, researchers analyzed whether a marker found at a unique site in chromosomal DNA was statistically associated with a disease or a disease phenotype. That is, was marker X (which usually did not encode a gene) found in all (or most) individuals who showed a specific disease or phenotype? If it did, then genes near the marker were proposed to contribute to or cause the disease.

This seminal paper spawned modern genetic analyses of disease.

1. **Botstein D, White RL, Skolnick M, Davis RW. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms.** *Am J Hum Genet* **32, 314 - 331**

Chronic diseases are caused by common variants of common genes. Since the Botstein et al method successfully identified chromosomal regions that caused monogenic diseases – an example would be Huntington's disease – it was modified in ways to identify the causal gene within the region. This was done by (i) sequencing the same region in many individuals suffering from the disease and comparing that sequence with individuals not showing signs of the disease or (ii) guessing which genes should be sequenced. The latter was more often used compared to comprehensive sequencing because of the cost of DNA sequencing. The Huntington chorea gene and the breast cancer genes (*BRCA1* and *BRCA2*) were found using these methods.

However, this success also directed much research for identifying genes involved in chronic diseases such as diabetes and obesity. That is, most of the genetic research was focused on discovering "mutant" genes at a specific locus, an understandable approach given the knowledge that mutated genes often cause disease. However, the failure to find such genetic defects in patients with chronic diseases led to the subsequent understanding embodied in the common disease/common variant hypothesis. That is, common variants of normal genes were responsible for chronic diseases. This hypothesis is largely responsible for the current genome-centric approach to the study of chronic diseases.

2. **Lander ES. 1996. The new genomics: global views of biology.** *Science* **274, 536 – 539**

3. **Collins FS, Guyer MS, Charkravarti A. 1997. Variations on a theme: cataloging human DNA sequence variation. Science 278, 1580 - 1581**

Identification of multiple chromosomal regions involved in chronic disease: Identifying the many genes that contribute to a chronic disease proved challenging in laboratory animals and in humans. However, Todd and his collaborators adapted quantitative trait loci (QTL) analyses developed for plants to laboratory animals, and specifically the nonobese diabetes (NOD) strain. NOD mice are a model of Type 1 diabetes. T1DM is thought to be a "single gene" disease. Todd and coworkers showed that multiple regions of the genome contribute to the development and severity of this disease. This paper is, to our knowledge, the first application of QTL analyses to inbred strains to identify disease QTLs, although Lander and Botstein described a method for QTL analyses with RFLPs in 1989.

4. **Risch, N, Ghosh, S, and Todd, JA. 1993. Statistical Evaluation of Multiple-Locus Linkage Data in Experimental Species and Its Relevance to Human Studies: Application to Nonobese Diabetic (NOD) Mouse and Human Insulin-dependent Diabetes Mellitus (IDDM).** *Am. J. Human Genet.* **53, 702 – 714.**

Among the problems of using association studies is the lack of evenly spaced markers and the regions identified may be quite large – between 1,000,000 and 40, 000,000 bp. The SNP consortia have (and will perfect) a set of SNPs evenly spaced at about one per 100,000 bp for mapping single gene defects and for use in QTL analyses.

The results: Most of the successes using these techniques were of "single" gene diseases – that is, diseases caused by a catastrophic mutation such as cystic fibrosis. To date, almost 1000 human disease genes have been identified and partially characterized — 97% of these genes are now known to cause monogenic diseases.

5. **Jimenez-Sanchez G, Childs B, Valle D. 2001 Human disease genes.** *Nature* **409, 853 - 855**

6. **Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. 2002. A comprehensive review of genetic association studies.** *Genet Med* **4, 45 – 61**

Environment affects expression of QTL producing complex phenotype: QTL analyses began in the plant research community. Paterson et al examined various phenotypes of tomato plants grown in two locations in California and in Israel. They demonstrated that the environment (in this case, physical location) changed the regions identified by QTL analyses, and the amount each contributed to a complex phenotype. Specifically, 29 QTL were identified, and only 4 were detected in the three environments tested, 10 in two environments, and 15 in only a single environment. "The two California environments were most similar, sharing 11/25 (44%) QTLs, while the Israel environment was quite different, sharing 7/20 (35%) and 5/26 (19%) QTLs with the respective California environments." Although this paper predates many studies identifying QTLs in human and model animal systems, many papers provide only a cursory description of the environment, especially concerning dietary variables. This becomes important for laboratory animals since ingredients in commercial chow diets are variable (see above).

7. **Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD, Lincoln SE, Lander ES, Tanksley SD. 1991. Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments.** *Genetics* **127, 181 - 97**

Some question gene X environment interactions: The thesis of our research is that some genes are regulated by diet, some by genotype, and some by the interaction of diet and genotype. We and now many others have experimental evidence to support that hypothesis. Others in the genetic epidemiology would agree (see Willett article). At least one group questions genotype X environment interactions.

8. **Clayton D, McKeigue PM. 2001. Epidemiological methods for studying genes and environmental factors in complex diseases.** *Lancet* **358, 356 - 360**

Note: Our 1994 paper, published by the University of Illinois Press in a volume dedicated to Willard Visek, and our 1997 JN paper, outlined an hypothesis similar to the CD/CV hypothesis since we proposed to identify genes underlying genetic susceptibility to disease in normal (i.e., not diseased) mice. However, unlike the gene-centric proposal of the CD/CV hypothesis, our contribution also includes environmental variables. This is a critical and important difference since DNA sequences do not exist in a vacuum, but rather interact with the environment. Diet is the most important environmental component since animals must eat to survive.

F. Combining QTLs and Gene Expression (but no diet)

Several researchers are beginning to combine genetics and gene expression to identify genes contributing to disease. Aitman et al a gene (CD36 – the fatty acid translocator) involved in defective fatty acid and glucose metabolism in hypertensive rats. Note, they did not do a genome wide expression analyses, but only examined the genes within the suspect QTL. No diets were described. A second paper used a genome-wide, expression and QTL analyses to discover interacting genes. This may become a powerful method for analyses but is costly since gene expression must be measured in a many F2 mice (each of which differs from other F2 mice in the population generated) to improve the statistical validity of the data. In addition, no diets were studied.

1. **Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. 1999. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats.** *Nat Genet* **21, 76 – 83**

2. **Schadt, EE, Monks, SA, Drake, TA, Lusis, AJ, Che, N, Colinayo, V, Ruff, TG, Milligan, SB, Lamb, JR, Cavet, G, Linsley, PS, Mao, M, Stoughton, RB, Friend, SH. 2003. Genetics of gene expression surveyed in maize, mouse and man.** *Nature* **422, 297 – 302**

G. Summary

The unique feature of our method is that we combine these separate disciplines into a common method and strategy (Figures 2 and 3). Individual steps of this strategy are practiced by many laboratories, but no report has appeared that combines these steps. It is often the case that genetic experiments do not use diet as a variable, or alternatively, nutritional experiments rarely analyze or assess genetic differences among study participants. Hence, our protocol is the only strategy that identifies genes regulated by diet, genotype, and genotype X diet interactions.
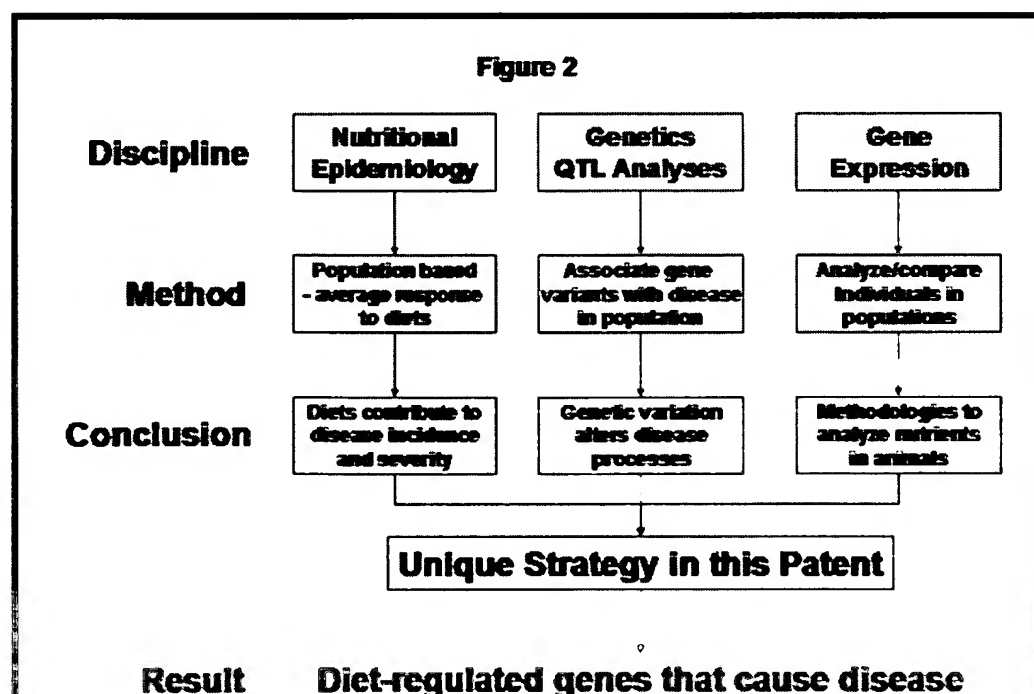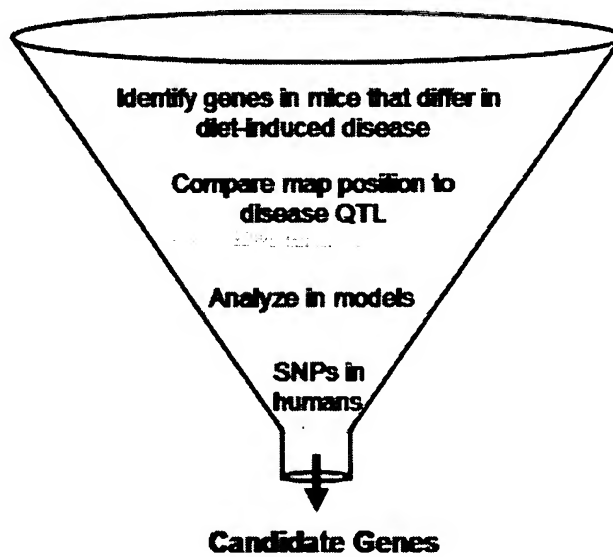

Figure 2

Discipline	Nutritional Epidemiology	Genetics QTL Analyses	Gene Expression
Method	Population based - average response to diets	Associate gene variants with disease in population	Analyze/compare individuals in populations
Conclusion	Diets contribute to disease incidence and severity	Genetic variation alters disease processes	Methodologies to analyze nutrients in animals

Unique Strategy in this Patent

Result   Diet-regulated genes that cause disease

Figure 3

Steps to Identify Diet-Regulated Genes

Identify genes in mice that differ in diet-induced disease

Compare map position to disease QTL

Analyze in models

SNPs in humans

Candidate Genes

Confidential NutraGenomics

APPENDIX B

RESPONSE BY THE INVENTOR, Dr. JAMES KAPUT, TO REQUEST FOR INFORMATION BY THE EXAMINER

Summary of Primary Papers Supporting Patent Application

Elliott, TS, Swartz, DA, Paisley, EA, Mangian, HJ, Visek, WJ, and Kaput, J. 1993. F_1F_O ATPase Subunit e Gene Isolated in a Screen for Diet-Regulated Genes. *Biochem. Biophys. Res. Comm.* **190, 167 - 174.**

This paper describes the cloning of two genes using (at the time) standard molecular biology methods. The uniqueness of this paper was that genes regulated by levels of dietary fat were used to identify unknown genes rather than examining absence or presence of a dietary component. One of the goals of this research was to identify genes regulated by dietary fat levels and then study them in promotion of breast cancer. Over 60 years of laboratory animal studies and many but not all human epidemiological studies link high fat diets to promotion of breast cancer. Note: only one strain (BALB/c) was used in this first analyses but this strain was known to have a high spontaneous incidence of breast cancer. We also used defined diets, rather than chow based diets, in our experiments, a typical method used in research conducted by our collaborator, Willard Visek.

Several laboratories were examining regulation of known genes by dietary factors but only Cousin's laboratory was interested in unknown genes. Cousin's group was identifying intestinal genes response to zinc. Our report and their lab's report were essentially published simultaneously. The research for this report was based upon concepts in the following articles. Note that references in the BBRC paper cite other specific contributions.

1. Tannenbaum, A. 1942. The Genetics and Growth of Tumors: II. Effects of Caloric Restriction per se. *Cancer Research* 2, 460 – 468. Genetic Basis of Disease

2. Tannenbaum, A. 1942. The Genetics and Growth of Tumors: III. Effects of a High Fat Diet. *Cancer Research* 2, 468 – 475. Genetic Basis of Disease

3. Hoag, WG. 1963. Spontaneous Cancer in Mice. Annals NY Acad. Sci 108, 805 – 831.

4. Coleman, DL. Kuzava, JE., and Leiter, EH. 1990. Effect of Diet on Incidence of Diabetes in Nonobese Diabetic Mice. *Diabetes* 39, 432 – 436.

5. Lardinois, CK, Caudill, T., Starich, GH. 1989. Dissimilar Fatty Acid Composition of Standard Rat Chow. *Am. J.Med.Sci.* 298, 2305 - 308.

6. Thigpen, JE, Setchell, KDR, Ahlmark, KB, Locklear, J., Spahr, T, Caviness, GF, Goelz, MF, Haseman, JK, Newbold, RR, and Forsythe, DB 1999. Phytoestrogen Content of Purified, Open- and Closed-Formula Laboratory Animal Diets. *Laboratory Animal Sci.* 49, 530 – 536.

7. Morris SM Jr, Nilson JH, Jenik RA, Winberry LK, McDevitt MA, Goodridge AG. 1982. Molecular cloning of gene sequences for avian fatty acid synthase and evidence for nutritional regulation of fatty acid synthase mRNA concentration. *J Biol Chem* 257, 3225 - 3229

8. Shay NF, Cousins RJ. 1993. Cloning of rat intestinal mRNAs affected by zinc deficiency *J. Nutrition* 123, 35 - 41

Although the following three papers were published after our first report, they support our hypothesis that dietary chemicals may affect gene expression directly:

1. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* 83, 803 - 812

2. Kliewar, SA, Lenhard, JM, Willson, TM, Patel, I, Morris, DC, and Lehmann, JM. 1995. A Prostaglandin J_2 Metabolit Binds Peroxisome – Activated Receptor γ and Promotes Adipocyte Differentiation. *Cell* 83, 813 – 819

3. Willett WC. 2002. Balancing life-style and genomics research for disease prevention. *Science* 296, 695 – 698 A review.

Kaput, J, Swartz, DA, Paisley, EA, Mangian, HJ, Daniel, W.L., Visek, WJ, and Kaput, J. 1994. Diet-Disease Interactions at the Molecular Level: An Experimental Paradigm. Presented at the 1993 Experimental Biology Fatty Acids and Gene Expression Symposium. *Journal of Nutrition* 124, 1296S - 1305S.

This theory and experimental paper joined molecular biology data (i.e., gene expression) with genetic information that maps a trait or disease to a specific chromosomal region. At the time of our publication, restriction fragment length polymorphisms (RFLP) were used to map human and other complex genomes. A variety of genetic and RFLP methods were used to map disease traits. Many of these disease or trait loci were large regions of the chromosome encoding many genes. Identifying the causal gene within the region was often difficult. Mapping diet regulated genes to a chromosomal locus would allow the linkage of our experimental data to known diseases.

The e subunit gene was linked to the *fat* locus on chromosome 8. Subsequent experiments from our laboratory showed that the e subunit gene did not encode the *fat* gene. To our knowledge, this was the first report that associated gene expression with genetic data. This report was based upon our previous research and the emerging field of mapping the human genome.

1. Botstein D, White RL, Skolnick M, Davis RW. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet* 32, 314 – 331

Our report discussed how different alleles of genes might contribute to atherosclerosis in BALB/c (intermediate risk) vs C57BL/6 (highly susceptible) vs C3H (resistant) differently depending upon the *Lfm1* (i.e., the e subunit gene) alleles present and the genetic background of the strains, a phenomenon called epistasis. Two theory papers supported our concepts that different alleles would contribute differently to common diseases such as atherosclerosis, obesity, diabetes. Note, however, that our proposals included the genotype X environment interaction control of regulation by dietary components. That is, different alleles of genes would be regulated differently by dietary components and this differential regulation might influence the contribution of different alleles to disease incidence and severity:

1. Lander ES. 1996. The new genomics: global views of biology. *Science* 274, 536 – 539

2. Collins FS, Guyer MS, Charkravarti A. 1997. Variations on a theme: cataloging human DNA sequence variation. Science 278, 1580 - 1581

Swartz, DA, Park, EI, Visek, WJ, and Kaput, J. 1996. The e Subunit of Murine F1F0-ATP Synthase: Genomic Sequence, Chromosomal Position, and Diet-Regulation. *Journal of Biological Chemistry* 271, 20942 - 20948.

Paisley, EA, Park, EI, Swartz, DA, Mangian, HJ, Visek, WJ, and Kaput, J. 1996. Temporal-Regulation of Serum Lipids and Stearoyl CoA Desaturase and Lipoprotein Lipase mRNA in BALB/cHnn Mice. *Journal of Nutrition* 126, 2730 - 2737.

These two papers show the importance of regulating time of feeding (Figure 3) relative to analyses of mRNA levels and other physiological measures such as serum lipid levels. Although this type of information was known for physiological factors, the importance of nutritional status on gene regulation is still not well known or commonly practiced. Since each diet-regulated gene may have a unique time course in response to eating and to the specific components of the diet, using a defined time and nutritional status for mRNA analyses is essential for comparing across experiments and dietary treatments. It should be noted that the use of a 2 hr time point for the fed nutritional state and 12 hr for the fasted state (used in subsequent experiments) is arbitrary. This is similar to the practice of using a 12 hour fast for human blood analyses.

Park, EI, Paisley, EA, Mangian, HJ, Swartz, DA, Wu, M, O'Morchoe, PJ, Behr, SA, Visek, WJ, and Kaput, J. 1997. Diets alter stearoyl CoA desaturase mRNA abundance differently in mice with different susceptibilities to diet-influenced diseases. *Journal of Nutrition* **127, 566 - 573.**

Stearoyl CoA desaturase was measured in two strains of mice with different susceptibilities to diet-induced disease. Others had examined gene expression in multiple strains but not with different types and levels of dietary fat. Note that this experiment did not seek to identify novel genes, but rather tested whether gene expression would differ by strain, type of diet, and level of dietary fat. This report was also among the first to correlate gene expression differences with subphenotypes (e.g., serum HDL-cholesterol) of a chronic disease such as atherosclerosis.

Kaput, J, Klein, KG, Reyes, EJ, Kibbe, WA, Visek, WJ, and Wolff, G. 2004. Identification of Genes Contributing to the Obese Yellow A^{vy} phenotype: Caloric Restriction, Genotype, Diet x Genotype Interactions. *Physiological Genomics* **18, 316-324**

This report describes the research supporting the patent application. It combines the knowledge generated by our previous publications and uses high throughput analyses (gene expression) to identify novel genes regulated by diet, genotype, and diet X genotype.

The strains used were obese yellow (A^{vy}/A) a model for obesity, type 2 diabetes, and breast cancer and Agouti (*A/a*) its "normal" control. The diets used for this study were 100% calories and 70% calories – that is, a caloric restriction model (Figures 1 - 4). We compared genes regulated by diet, genotype, and genotype X diet to quantitative trait loci published by others to identify candidate genes (Figure 5). These genes are likely to cause or contribute to chronic disease since they are regulated by a genotype known to increase disease incidence and severity, or by a diet known to increase disease incidence or severity, or by an interaction between diet and genotype.

Although gene expression has been widely used in the past few years, our combination of strains differing in disease susceptibility and diets known to induce different disease outcomes is the unique feature of our experimental design. Identifying novel genes in a defined genotype will aid in understanding the genetic and environmental basis of complex disease development and progression. This work approach is important because most known human disease genes cause monogenic diseases – that is, diseases caused by a mutation in a single gene. In addition, genetic association studies have failed to identify the genes that contribute to chronic diseases. This failure may be explained by the genetic heterogeneity of humans which produces epistatic (gene-gene) interactions and by the diverse diets of humans which are typically not controlled in genetic epidemiology studies (in addition to experimental design issues).
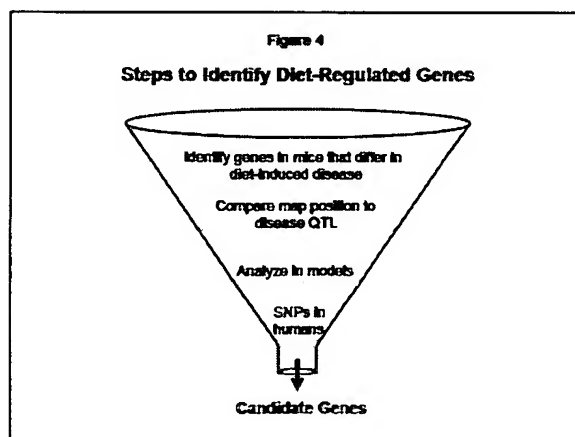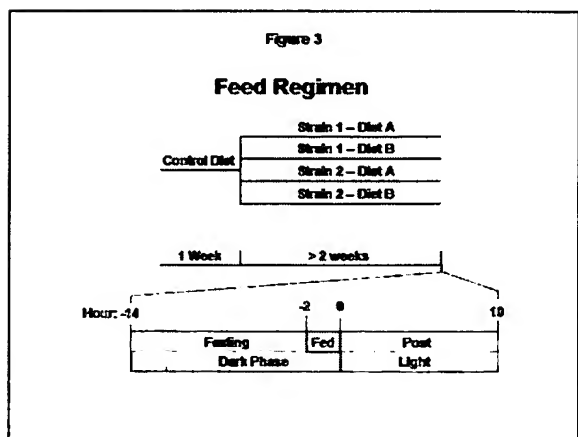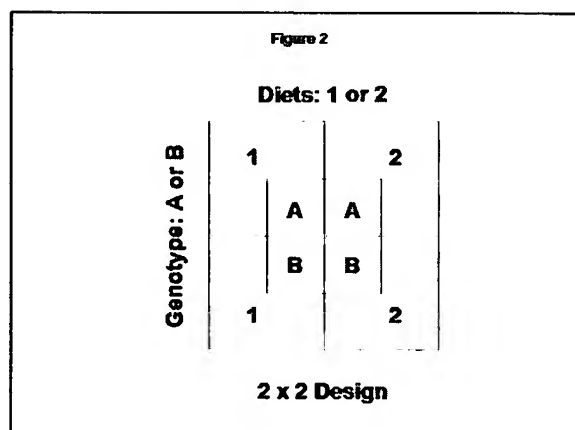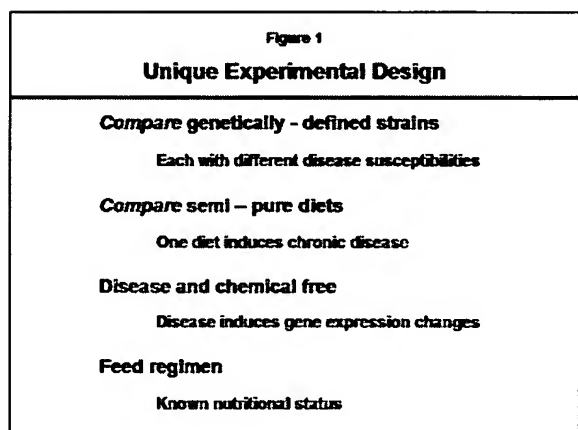
Our protocol (Figures 1 - 5) is the only one that identifies genes regulated by diet, by genotype, and by diet X genotype interactions at the same time (Figure 6). Such knowledge is important for designing human studies to examine gene-disease and gene – diet associations since we can predict environmental influences on each gene and, with the appropriate strains, predict epistatic interactions.

1. Risch, N, Ghosh, S, and Todd, JA. 1993. Statistical Evaluation of Multiple-Locus Linkage Data in Experimental Species and Its Relevance to Human Studies: Application to Nonobese Diabetic (NOD) Mouse and Human Insulin-dependent Diabetes Mellitus (IDDM). *Am. J. Human Genet.* 53, 702 – 714.

2. Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD, Lincoln SE, Lander ES, Tanksley SD. 1991. Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. *Genetics* 127, 181 - 97

3. Clayton D, McKeigue PM. 2001. Epidemiological methods for studying genes and environmental factors in complex diseases. *Lancet* 358, 356 - 360

4. Jimenez-Sanchez G, Childs B, Valle D. 2001 Human disease genes. *Nature* 409, 853 - 855

5. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. 2002. A comprehensive review of genetic association studies. *Genet Med* 4, 45 – 61

Several researchers are beginning to combine genetics and gene expression to identify genes contributing to disease. Aitman et al a gene (CD36 – the fatty acid translocator) involved in defective fatty acid and glucose metabolism in hypertensive rats. Note, they did not do a genome wide expression analyses, but only examined the genes within the suspect QTL. No diets were described. A second paper used a genome-wide, expression and QTL analyses to discover interacting genes. This may become a powerful method for analyses but is costly since gene expression must be measured in a many F2 mice (each of which differs from other F2 mice in the population generated) to improve the statistical validity of the data. In addition, no diets were studied.

1. Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. 1999. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* 21, 76 – 83

2. Schadt, EE, Monks, SA, Drake, TA, Lusis, AJ, Che, N, Colinayo, V, Ruff, TG, Milligan, SB, Lamb, JR, Cavet, G, Linsley, PS, Mao, M, Stoughton, RB, Friend, SH. 2003. Genetics of gene expression surveyed in maize, mouse and man. *Nature* 422, 297 – 302
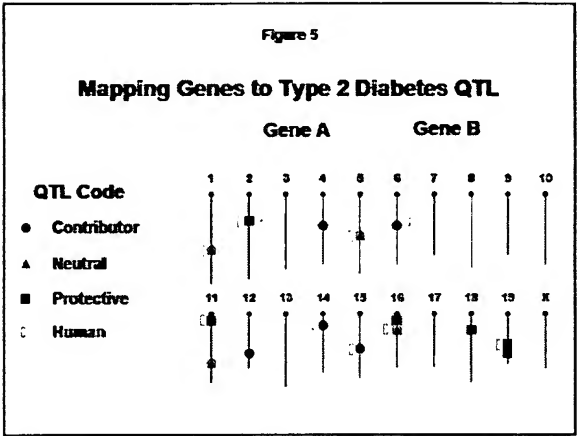
Figure 1
Unique Experimental Design

Compare genetically - defined strains
   Each with different disease susceptibilities

Compare semi – pure diets
   One diet induces chronic disease

Disease and chemical free
   Disease induces gene expression changes

Feed regimen
   Known nutritional status

Figure 2

Diets: 1 or 2

Genotype: A or B

1			2
	A	A	
	B	B	
1			2

2 x 2 Design

Figure 3
Feed Regimen

Control Diet
Strain 1 – Diet A
Strain 1 – Diet B
Strain 2 – Diet A
Strain 2 – Diet B

1 Week | > 2 weeks

Hour: -14   -2   0   10

Fasting | Fed | Post
Dark Phase | Light

Figure 4
Steps to Identify Diet-Regulated Genes

Identify genes in mice that differ in diet-induced disease

Compare map position to disease QTL

Analyze in models

SNPs in humans

Candidate Genes

Figure 5

Mapping Genes to Type 2 Diabetes QTL

Gene A　　　Gene B

QTL Code
- ● Contributor
- ▲ Neutral
- ■ Protective
- ⊏ Human

Figure 6

Identifying Candidate Genes

Types of Disease Genes

Method	Genotype	Diet	Genotype X Diet	Example
Genetic	✓	–	–	BRCA1 (Breast cancer)
Models (cells)	✓	–	–	Insulin Receptor
Nutritional	–	✓	–	Cholesterol
NutraGenomics	✓	✓	✓	Complete set

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**RESPONSE BY THE INVENTOR, Dr. JAMES KAPUT, TO
REQUEST FOR INFORMATION BY THE EXAMINER**

NutraGenomics

Review of Key Publications

Jim Kaput, PhD.
NutraGenomics, Inc
7 August 2002

NutraGenomics combines molecular genetic technologies and approaches to analyze the response to well-balanced diets (nutrition) in inbred mice with different susceptibilities to disease (comparative genomics).

Genetics – Experimental use of Comparative Genomics

Comparative genetics has been an active field for almost 100 years. Much of the work was initiated by C.C. Little and continued by scientists at the Jackson Laboratory. Finding the "first" article describing such approaches is problematic because the field of mouse genetics began in the early 1900s.

Strain differences reveal information about diseases processes: The Hoag paper is a comprehensive review of all literature related to cancer in inbred strains dating to the early part of the 1900s, and demonstrates that strain specific differences exist. Hoag cites studies that compared strains in one study.

1. Hoag, WG. 1963. Spontaneous Cancer in Mice. Annals NY Acad. Sci 108, 805 – 831.

The effect of an individual gene on physiology differs depending upon the genetic background: A review of Coleman's classic work on two single gene mutations, obese (*ob*) and diabetes (*db*). His work showed that these genes affect different inbred strains differently. Specifically, BL/6 inbred mice respond to *ob* and *db* genes by expanding beta cell populations and the increased cell numbers eventually produce enough insulin to control hyperglycemia. In BL/Ks inbred mice carrying either *ob* or *db*, beta cell expansion doesn't occur and full blown diabetes develops. The genetic backgound (i.e., in this case, BL/Ks) alters expression – or more precisely – the effect of individual mutant genes.

2. Coleman, DL. 1978. Obese and Diabetes: Two Mutant Genes Causing Diabetes-Obesity Syndromes in Mice. *Diabetologia* **14**, 141 – 148 (1978) A review article.

Importance of Diet Consistency on Model Studies and Disease - Experimental

We take great care in designing and feeding the mice – in an attempt to control nutrition and nutritional status. These papers demonstrate the importance of that approach.

Altering diet changes incidence of diabetes in NOD mice. Much of the research on laboratory animal strains is done with chow diets – diets from a commercial source made from least expensive, unrefined material. Colman et al showed that a semi-defined diet – one in known quantities and qualities of dietary constituents were mixed – produced different incidence of diabetes in NOD mice. The results are dramatic: animals fed chow diets had a much greater incidence of diabetes compared to mice fed the semi-defined diets.

We have GC data from a collaborator that analyzed chow diets similar to that used in the Colman study compared to a semi-defined diet, and believe that the difference is mainly in STEROLS and STEROL-DERIVATIVES. We have not had an opportunity to test this hypothesis, but it has direct relevance to INITIATION of Type 1 diabetes.

3.   Coleman, DL. Kuzava, JE., and Leiter, EH. 1990. Effect of Diet on Incidence of Diabetes in Nonobese Diabetic Mice. *Diabetes* **39**, 432 – 436.

Constituents in Diets Vary, unless Rigorously Controlled. These two papers are examples of research on the variability of chow diets. I often cite two others – one for toxicology experiments and one for pharmacology experiments. A closed-formula diet report the dietary ingredient, but the concentrations are not stated and may vary from batch to batch. Open-formula diets should report ingredients, concentrations, and these should not vary from batch to batch.

Both of these studies demonstrate a significant variation in constituents and concentrations between diets and/or lots. Note, that this variation may not matter for certain biological experiments, but will be very important for studies of gene regulation – particularly when comparing across different feeding studies. We use diets constructed by Research Diets Inc (New Brunswick, NJ) and they are rigorously controlled. In addition, we also have adopted a 4% corn oil diet as a control in all feeding experiments, allowing for meaningful cross experiment comparisons.

Not worth a citation – but our regimen for making sure the animals are in one nutritional state is a corollary concern for gene expression studies.

4.   Lardinois, CK, Caudill, T., Starich, GH. 1989. Dissimilar Fatty Acid Composition of Standard Rat Chow. *Am. J.Med.Sci.* **298**, 2305 - 308.

5.   Thigpen, JE, Setchell, KDR, Ahlmark, KB, Locklear, J., Spahr, T, Caviness, GF, Goelz, MF, Haseman, JK, Newbold, RR, and Forsythe, DB 1999. Phytoestrogen Content of Purified, Open- and Closed-Formula Laboratory Animal Diets. *Laboratory Animal Sci.* **49**, 530 – 536.

Nutrient Control of Gene Expression

Our underlying assumption is that a subset of genes regulated by diet are involved in disease processes.

Gene Expression – Single Genes: Many researchers investigate the nutritional regulation of specific genes. The vast majority of these studies pre-select the gene of interest based upon historical or biochemical rationale. For example, Goodridge's lab published 23 papers on various aspects of fatty acid and TCA cycle enzymes and then continued that work through the cloning era. This appears to be the first paper using molecular biology methods to examine expression of a single gene in response to dietary changes.

For the purposes of the patent, changes in expression of a single gene rarely produce CHRONIC disease. Over-expression of certain oncogenes do alter phenotype (of course), but these are typically caused by mutations, not naturally occurring allelic variants.

6. Morris SM Jr, Nilson JH, Jenik RA, Winberry LK, McDevitt MA, Goodridge AG. 1982. Molecular cloning of gene sequences for avian fatty acid synthase and evidence for nutritional regulation of fatty acid synthase mRNA concentration. *J Biol Chem* **257**, 3225 - 3229

The transition to high throughput methods to isolate previously unidentified diet regulated genes: With the advent of differential screening technologies, it was possible to identify and characterize previously unknown genes regulated by dietary variables. The first two papers using this approach were for isolating genes regulated by dietary lipids (our 1993 paper) and by dietary zinc – both were published in January 1993. The purpose of Cousins' paper was to isolate Zn transporter genes. Cousins group went on to use differential display technologies to identify other Zn regulated genes (as did we, but we did not publish the work). Modern techniques of course, use microarray analyses.

7. Shay NF, Cousins RJ. 1993. Cloning of rat intestinal mRNAs affected by zinc deficiency *J. Nutrition* **123**, 35 - 41

Dietary chemicals control gene expression. Many reports in the late 1980s and early 1990s showed regulation of gene expression by diet, but many interpreted the regulation as going through hormonal pathways and signaling (e.g., insulin and glucagons regulation). The PPAR story broadened the possibilities of gene regulation to include specific dietary chemicals.

PPARs were discovered by Gustafsson's group in Stockholm and are dubbed orphan receptors - that is, their activation ligands have not been identified. These receptors belong to a nuclear receptor superfamily, a group of structurally related, ligand-dependent transcription factors. There are about 50 such similar genes and the ligands for many are still uncharacterized. Evans' group identified the first ligand for the nuclear receptor, Peroxisome Proliferator Activated Receptor – gamma (PPAR-γ), an arachidonate metabolite, 15-deoxy-delta 12, 14-prostaglandin J2. Lehmann published essentially the same results in the same issue of *Cell*. Figure 1 shows the arachidonic acid pathway, although it doesn't include the J2 derivative. This

Figure 1
Arachidonic Acid metabolism
(from Sigma Aldrich)
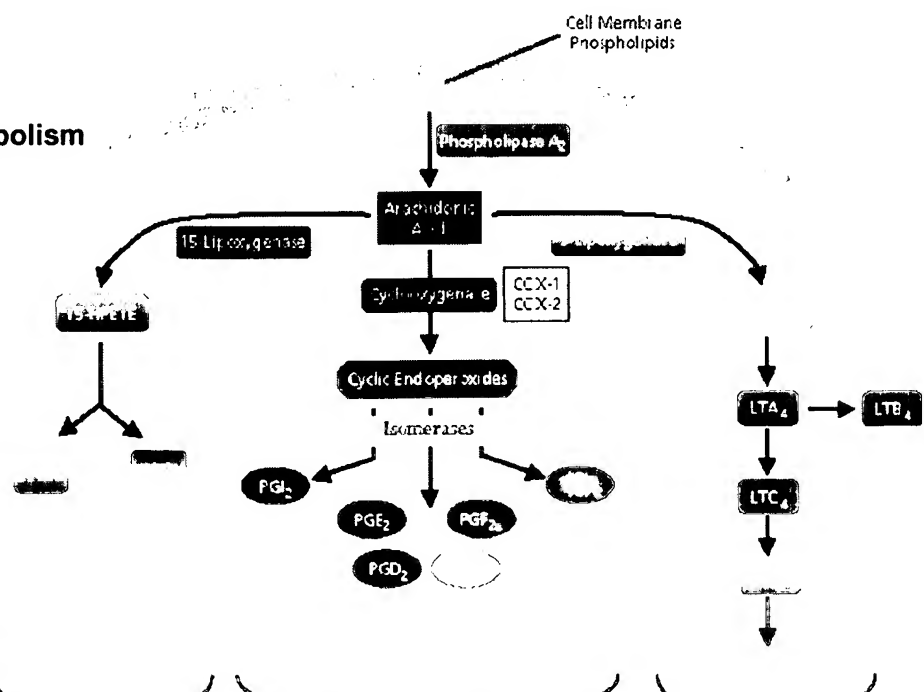
chemical activates PPAR-γ and induces adipogenesis. Many of the orphan receptors are likely to be activated by ligands derived from the diet. For example, LXR is a liver specific oxysterol receptor that regulates cholesterol metabolism, and FXR (farnesoid X receptor) is a regulated by bile acids.

8. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 1995. 15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma. *Cell* **83**, 803 - 812

9. Kliewar, SA, Lenhard, JM, Willson, TM, Patel, I, Morris, DC, and Lehmann, JM. 1995. A Prostaglandin J$_2$ Metabolit Binds Peroxisome – Activated Receptor γ and Promotes Adipocyte Differentiation. *Cell* **83**, 813 – 819

Diet Effect on Disease - Experimental

Numerous publications look at the effect of diet on health in mice or laboratory animals. The first study showing that dietary fat promoted tumors in laboratory animals was published in the 1940s. Since then, many studies have demonstrated an effect of diet on disease in laboratory animals for virtually every chronic diseases.

Diet influences health: Based upon numerous historical reviews, this is the first paper describing the effects of high fat diets on cancer and a more recent review of the effect of diet on health.

10. Tannenbaum, A. 1942. The Genetics and Growth of Tumors: III. Effects of a High Fat Diet. *Cancer Research* **2**, 468 – 475. Genetic Basis of Disease

11. Willett WC. 2002. Balancing life-style and genomics research for disease prevention. *Science* **296**, 695 – 698 A review.

Genetic Effects on Disease

The foundation for the modern approach to the analyses of genetic basis of disease was proposed by Botstein, White, Skolnick, and Davis in 1980. Although methods for analyses of polymorphisms have improved, they are all based upon the same concept: associate variations in sequence to complex single gene defects (cystic fibrosis) or complex phenotypes.

This seminal paper spawned modern genetic analyses of disease.

12. Botstein D, White RL, Skolnick M, Davis RW. 1980. Construction of a genetic linkage map in man using restriction fragment length polymorphisms. *Am J Hum Genet* **32**, 314 - 331

Chronic diseases are caused by common variants of common genes. Most of the genetic research was focused on discovering "mutant" genes at a specific locus, an understandable approach given the knowledge that mutated genes often cause disease. However, the failure to find such genetic defects in patients with chronic diseases led to the subsequent understanding embodied in the common disease/common variant hypothesis. That is, common variants of normal genes were responsible for chronic diseases. This hypothesis is largely responsible for the current genome-centric approach to the study of chronic diseases.

13. Lander ES. 1996. The new genomics: global views of biology. *Science* **274**, 536 – 539

14. Collins FS, Guyer MS, Charkravarti A. 1997. Variations on a theme: cataloging human DNA sequence variation. Science **278**, 1580 - 1581

Note: Our 1994 paper, published by the University of Illinois Press in a volume dedicated to Willard Visek, and our 1997 JN paper, outlined an identical hypothesis since we proposed to identify genes underlying genetic susceptibility to disease in normal (i.e., not diseased) mice. Our contribution also includes environmental variables.

Identification of multiple chromosomal regions involved in chronic disease: NOD mice are a model of Type 1 diabetes. The impression was that it was a "single gene" disease. Todd and coworkers showed that multiple regions of the genome contribute to the development and severity of this disease. I believe it is the first application of QTL analyses to inbred strains to identify disease QTLs, although Lander and Botstein described a method for QTL analyses with RFLPs in 1989.

15. Risch, N, Ghosh, S, and Todd, JA. 1993. Statistical Evaluation of Multiple-Locus Linkage Data in Experimental Species and Its Relevance to Human Studies: Application to Nonobese Diabetic (NOD) Mouse and Human Insulin-dependent Diabetes Mellitus (IDDM). *Am. J. Human Genet.* **53,** 702 – 714.

Among the problems of using association studies is the lack of evenly spaced markers. The SNP consortia have (and will perfect) a set of SNPs evenly spaced at about one per 100,000 bp for mapping single gene defects and for use in QTL analyses.

The results: Most of the successes using these techniques were of "single" gene diseases – that is, diseases caused by a catastrophic mutation such as cystic fibrosis. To date, almost 1000 human disease genes have been identified and partially characterized — 97% of these genes are now known to cause monogenic diseases.

16. Jimenez-Sanchez G, Childs B, Valle D. 2001 Human disease genes. *Nature* **409**, 853 - 855

17. Hirschhorn JN, Lohmueller K, Byrne E, Hirschhorn K. 2002. A comprehensive review of genetic association studies. *Genet Med* **4**, 45 – 61

Environment affects expression of QTL producing complex phenotype: QTL analyses began in the plant research community. The Paterson paper examined various phenotypes of tomato plants grown in two locations in California and in Israel. They demonstrated that the environment (in this case, physical location) changed the regions identified by QTL analyses, and the amount each contributed to a complex phenotype. Specifically, 29 QTL were identified, and only 4 were detected in the three environments tested, 10 in two environments, and 15 in only a single environment. "The two California environments were most similar, sharing 11/25 (44%) QTLs, while the Israel environment was quite different, sharing 7/20 (35%) and 5/26 (19%) QTLs with the respective California environments." Although this paper predates many studies identifying QTLs in human and model animal systems, many papers provide only a cursory description of the environment, especially concerning dietary variables. This becomes important for laboratory animals since ingredients in commercial chow diets are variable (see above).

18. Paterson AH, Damon S, Hewitt JD, Zamir D, Rabinowitch HD, Lincoln SE, Lander ES, Tanksley SD. 1991. Mendelian factors underlying quantitative traits in tomato: comparison across species, generations, and environments. *Genetics* **127**, 181 - 97

Some question gene X environment interactions: The thesis of our research is that some genes are regulated by diet, some by genotype, and some by the interaction of diet and genotype. We have experimental evidence to support that hypothesis. Others in the genetic epidemiology would agree (see Willet article). At least one group questions genotype X environment interactions.

19.   Clayton D, McKeigue PM. 2001. Epidemiological methods for studying genes and environmental factors in complex diseases. *Lancet* **358**, 356 - 360

Combining QTLs and Gene Expression (but no diet)

The only paper that I am aware of that combines QTL analyses and expression analyses identified a gene (CD36 – the fatty acid translocator) involved in defective fatty acid and glucose metabolism in hypertensive rats. Note, they did not do a genome wide expression analyses, but only examined the genes within the suspect QTL. No diets were described.

20.   Aitman TJ, Glazier AM, Wallace CA, Cooper LD, Norsworthy PJ, Wahid FN, Al-Majali KM, Trembling PM, Mann CJ, Shoulders CC, Graf D, St Lezin E, Kurtz TW, Kren V, Pravenec M, Ibrahimi A, Abumrad NA, Stanton LW, Scott J. 1999. Identification of Cd36 (Fat) as an insulin-resistance gene causing defective fatty acid and glucose metabolism in hypertensive rats. *Nat Genet* **21**, 76 - 83